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DEVELOPMENT OF BIOCHEMICAL CYANIDE ANTIDOTES

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Twenty-four different substances were tested as potential cyanide antidotes using cultured rat pheochromocytoma (p-PC12) cells. Prevention of cyanide-induced biochemical changes (increased cell calcium, peroxide formation, cytochrome oxidase inhibition and inhibition of antioxidant defense enzymes) in isolated PC12 cells was used as a measure of *in vitro* antidotal effectiveness. For comparison, protection against cyanide lethality in mice (LD_{50}) was evaluated for ~11 compounds. The linear regression line relating *in vitro* with *in vivo* antidotal activity had a correlation coefficient of 0.54. Thus more data are needed to fully evaluate the *in vitro* system but this rapid screening procedure may prove to be an efficient way to predict whether a substance is a useful cyanide antagonist.

RA V; Lab Animals; Mice; Adrenal Medulla; Cell Culture; In Vitro Analysis; Cyanides; Antidotes; Therapy

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G.E. Isom (YES) 29 Nov 1990
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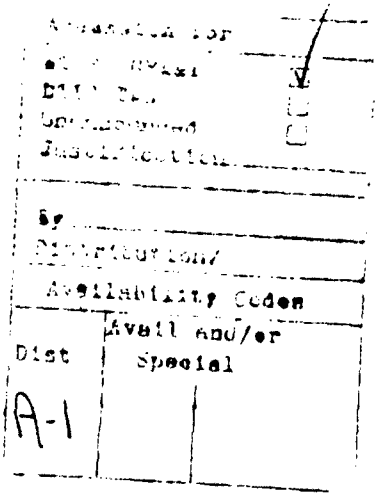

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INTRODUCTION

NATURE OF THE PROBLEM

Cyanide is notorious as a poison and ideal in some respects as a chemical weapon. It kills rapidly, dissipates quickly and leaves no toxic residue. Cyanide was used as a war gas in World War I (1) and in the Iran-Iraq War (2,3). It may be used not only in large scale warfare but also in local terrorist actions. Effective means of neutralizing the effects of cyanide should be available for protection of combat troops.

Standard antidotes for cyanide (sodium thiosulfate to accelerate conversion to thiocyanate and sodium nitrite to generate methemoglobin) have been available for many years. However, much of our knowledge of cyanide's actions is very recent and agents designed to counteract the biochemical changes caused by cyanide have not been extensively studied.

BACKGROUND OF PREVIOUS WORK

A recent report from our laboratory (4) shows that chlorpromazine, which is a cyanide antidote *in vivo* (5), blocks cyanide-induced lipid peroxidation and elevation of cytosolic calcium by cyanide. These results suggest that protection against biochemical changes induced by cyanide *in vitro* may provide antidotal effectiveness against cyanide *in vivo*. A brief review of cyanide-induced biochemical changes which are the basis for the *in vitro* screening procedure for cyanide antidotes is given below.

Blockade of mitochondrial cytochrome oxidase with depletion of cellular energy stores has long been considered the main biochemical effect of cyanide. However, recent reports cast doubt

on this concept. For example, Yamamoto (6) showed that mice rendered unconscious by cyanide showed no decrease in brain ATP whereas liver ATP decreased to 60% of control. Ballantyne (7) provides a good review of evidence for and against the concept that blockade of cytochrome oxidase is the critical lesion in cyanide poisoning. Thus cytochrome oxidase inhibition may explain only part of the toxic syndrome caused by cyanide.

Cyanide's effect on neural systems can be very rapid in onset. Atken and Braitman (8) using transverse slices of guinea pig hippocampus, found that application of cyanide (10 to 200 μM) almost immediately depressed synaptic transmission between Schaffer collateral commissural fibers and CA1 pyramidal cells. Direct electrical excitability of the axons was not affected since 500 μM cyanide had no effect on antidromic activation of pyramidal cells. Cyanide's effect reversed rapidly on washout suggesting that cyanide has a direct action on neurons not mediated by metabolic inhibition.

Not all neural systems are inhibited by cyanide. Persson et al., (9) found rapid release of brain neurotransmitters after ip injection of NaCN. Dopamine, for example, decreased in straitum within 60 seconds after cyanide administration. A related study (10) showed that cyanide released dopamine and glutamate from brain slices and from synaptosomes. Whether cyanide-induced neurotransmitter release is due to metabolic inhibition is not known. However, many symptoms of cyanide intoxication e.g. tremor and convulsions may be due to neurotransmitter release.

An important biochemical change after cyanide exposure involves calcium. Cyanide-induced tremors in mice parallel an increase in whole brain calcium (11). Both tremors and accumulation of brain calcium are diminished by pretreatment with diltiazem, a calcium channel blocker (11).

Elevated cytosolic calcium following cyanide exposure may generate peroxide radicals which lead to lipid peroxidation and cell damage (12). Diltiazem pretreatment blocks membrane lipid peroxidation in mouse brain caused by cyanide (12). Allopurinol, the xanthine oxidase inhibitor, also blocks cyanide-induced lipid peroxidation, which agrees with the suggestion that this enzyme is involved in generation of superoxide radicals after cyanide exposure (13).

If oxygen radicals mediate cell damage after cyanide exposure, then antioxidant systems in cells may be critical in protection against cyanide. However, enzymes which degrade oxygen radicals are also inhibited by cyanide (14). Suppression of antioxidant defences may be critical to cell damage caused by cyanide.

PURPOSE OF THE PRESENT WORK

The aim of this project is to evaluate an *in vitro* screening procedure for cyanide antidotes. The procedure involves inhibition of cyanide-induced biochemical changes in cultured neuronal-like cells ("PC12" cells from a rat adrenomedullary tumor) by potential antidotes. These cyanide-induced changes (increased cell calcium, lipid peroxidation, inhibition of cytochrome oxidase, catecholamine secretion and inhibition of antioxidant defenses) may mediate the toxic effects of cyanide in mammalian systems (4,7). Substances

which inhibit these biochemical changes may protect against cyanide intoxication. Evaluation of the screen involves correlation of effectiveness of substances which protect against cyanide in vitro with effectiveness of these same substances against cyanide toxicity in mice. It is hoped that active agents capable of preventing biochemical changes induced by cyanide in isolated cells may prove to be safe, effective antidotes for cyanide intoxication and enhance the action of traditional antidotes.

EXPERIMENTAL METHODS

Basically, attempts were made to block 5 different biochemical responses of PC12 cells to cyanide:

1. Inhibition of antioxidant defense enzymes:
   a) catalase
   b) superoxide dismutase
   c) glutathione reductase
2. Rise in intracellular free calcium
3. Peroxide generation
4. Cytochrome oxidase inhibition
5. Dopamine release

Chemicals selected as potential antidotes included anti-convulsants, adrenergic blockers, antiarrhythmics, antioxidants, sugars, antipsychotics, etc. Known cyanide antidotes were also included for comparative purposes and to allow a correlation to be drawn between in vitro effectiveness and antidotal potency in the mouse LD_{50}.

All potential antidotes at 10 μM concentrations, were added to suspensions of PC12 cells 15 min prior to cyanide. The

concentration of cyanide employed (usually 1 mM) was determined from dose-response curves. Results obtained with cyanide alone were compared with those obtained using cyanide plus the antidote. Controls were also run to determine resting activity of each of the above parameters. Experiments were generally repeated 2 to 4 times to establish the validity of the observations.

CELL CULTURE: PC12 cells (15) were obtained from American Type Culture Collection (Rockville, MD) and grown as monolayers in RPMI 1640 media supplemented with 10% v/v heat inactivated horse serum, 5% v/v fetal bovine serum and 1% penicillin-streptomycin in Falcon plastic tissue culture flasks. Heat inactivation was effected by heating at 56°C for 60 min. Cells cultured for 5 to 6 days after transfer were harvested from monolayers and suspended in Krebs-Ringer bicarbonate solution (125 mM NaCl, 5 mM KCl, 25 mM HEPES-NaOH, 6 mM glucose, 5 mM $NaHCO_3$, 1.2 mM $MgSO_4$, 1.2 mM KH_2PO_4 and 1 mM $CaCl_2$) at pH 7.42. Cell populations were estimated by counting or by weight and appropriate dilutions made.

ANTIOXIDANT DEFENSE ENZYMES

Catalase: Hydrogen peroxide is damaging to living systems, frequently because it gives rise to $OH^{\bullet}$ radicals which can produce alterations in DNA, lipids and proteins in cells. It is therefore important that cells limit H_2O_2 accumulation. Catalase mediates the conversion of H_2O_2 to H_2O and O_2. The enzyme is distributed throughout the body and may play an important role in limiting oxidative damage after cyanide exposure.

Enzyme activity was measured at 20°C in 50 mM sodium phosphate buffer at pH 7.0 using 50 mM H_2O_2 as substrate (16). Decomposition

of H_2O_2 by catalase was monitored by measuring change in extinction spectrophotometrically (Perkin Elmer Lambda 3B) at 240 nm for 3 min. Catalase activity was expressed as a first order rate constant (K).

Superoxide Dismutase (SOD): CuZu superoxide dismutase, which is the cyanide sensitive form, is found in virtually all animal cells and serves to catalyse breakdown of superoxide (O_2^-) to H_2O_2 and O_2. This is an important reaction because it prevents interaction of O_2^- with iron in the cell and blocks formation of the highly reactive hydroxyl radical ($OH^\bullet$) (17).

SOD enzyme levels were determined spectrophotometrically (Perkin Elmer Lambda 3B) at 560 nm by the nitro blue tetrazolium method in which total SOD activity in homogenates is estimated (18). SOD activity was expressed as amount of enzyme (1 unit) that inhibits reduction of nitroblue tetrazolium by 50%.

Glutathione Reductase: Like catalase, glutathione peroxidase mediates breakdown of H_2O_2. Although no inhibition of glutathione peroxidase in PC12 cells by cyanide was determined, glutathione reductase activity was reduced about 50% in this system by cyanide. Glutathione reductase restores glutathione to its reduced state after oxidation by glutathione peroxidase. Thus, glutathione is an important antioxidant which supports the activity of glutathione peroxidase. The reductase enzyme ensures an adequate supply of glutathione for glutathione peroxidase. Agents which protect the glutathione reductase may be valuable for preventing cyanide damage to cells.

Glutathione reductase was measured in cell homogenates containing 2 mM NADPH and 20 mM glutathione disulfide and monitoring the disappearance of NADPH at 340 nm (19) in a Perkin Elmer Lambda 3B spectrophotometer.

FREE INTRACELLULAR CALCIUM

Elevated intracellular calcium may damage cells by activation of proteases which can digest proteins within cells (20). Peroxide generation may also be favored by elevated cytosolic calcium. Blockade of cyanide-induced increases in cytosolic calcium may be one of the most important tests in the screening procedure.

The calcium-sensitive fluorescent dye Quin 2 AM was dissolved in 50 mM dimethylsulfoxide and stored at -20°C until used. Twenty µl of Quin 2 AM, 50 mM, was added to a 3 ml suspension of PC12 cells (~10^7 cells/ml) in loading buffer (RPMI medium containing 20 mM HEPES and 5 mM $NaHCO_3$, pH 7.4) and the mixture incubated at 37°C for 1 hr with frequent shaking. After 1 hr, cells were centrifuged (300 X g for 2 min) and resuspended in loading buffer to give 3 X 10^6 cells/ml. For determination of cytosolic calcium, 1 ml of the above cell suspension was spun at 5000 g for 8 sec. and resuspended in 1 ml of Krebs-Ringer Bicarbonate (KRB) buffer. Cells were washed two more times and then resuspended in 2 ml KRB solution in a quartz cuvette. The cuvette was maintained at 37°C and continuously stirred. Monochromotor settings were 339 nm excitation (2 mm slit) and 492 nm emission (10 mm slit). After each assay, triton X-100 (20 µl of 10% v/v) was added to lyse the cells and release Quin 2 (Fmax). EDTA (5 mM) and tris base (0.1 M)

were added to chelate calcium and give Fmin. Cytosolic calcium was then calculated using the following equation:

$[Ca^{2+}]$ = K and (F - Fmin)/Fmax -F

Kd = 115 nM

F = Fluorescence of sample

PEROXIDE GENERATION: The assay is based on conversion of 2,7-dichlorofluorescein to the fluorescent form of 2,7 dichloro-fluorescein by peroxides (21,22). PC12 cells were harvested and suspended in 10 ml of KRB solution, ten μl of 20 mM dye was added and kept at room temperature for 15 min. Then the cells were washed two times with KRB solution by centrifugation at 5000 g for 8 sec. After centrifugation, cells were resuspended in KRB solution to give 4 X 10^6 cells/ml. Fluorescence was measured in a Aminco Bowman Spectrofluorometer before and 5 min after addition of KCN (Excitation 475 nm, emission 525 nm). Results were expressed as μmoles H_2O_2 generated/10^7 cells.

Although H_2O_2 is used as the standard for the fluorescent assay of peroxide, other peroxides eg. lipid peroxides are also detected by the assay.

CYTOCHROME OXIDASE

Impaired energy metabolism probably explains many effects of cyanide. Any antidote which protects the enzyme cytochrome oxidase from inhibition by cyanide may be of great value in clinical treatment as well as in basic studies of cyanide's actions.

Cytochrome oxidase activity was determined spectrophotometrically by measuring the rate of oxidation of reduced cytochrome C (23-25). The incubation mixture was placed in

sealed cuvettes in which the incubation medium had been equilibrated with either air or 100% oxygen, and the determinations were conducted under the appropriate atmosphere at 25°C. The analytical procedure consisted of adding 2.4 ml of 30 mM Tris-HCl buffer (pH 7.4) to a 3 ml cuvette followed by 0.5 ml of reduced cytochrome C. The homogenate (0.1 ml) was added to the assay mixture and the decrease in absorbance at 550 nm was recorded over a 3-min period in a Perkin Elmer Lambda 3B Spectrophotometer. Finally, 0.1 ml of saturated potassium ferricyanide was added to oxidize completely the cytochrome C, and the absorbance was redetermined. Cytochrome oxidase activity was expressed as moles of cytochrome C oxidised/min/10^6 cells.

DOPAMINE RELEASE: Release of neurotransmitters by cyanide may be a critical aspect of the toxic syndrome and may explain many of the symptoms manifested after cyanide exposure. It was important therefore to test the effects of potential antidotes on cyanide-induced dopamine release from PC12 cells.

Dopamine released into the incubation buffer (KRB solution) of PC12 cells during 30 min incubation was estimated in the supernatant after centrifugation of the cells (5000 g for 8 sec). Analyses were conducted using high performance liquid chromatography (HPLC) with electrochemical detection (26). The HPLC system consisted of a 110B Beckman pump equipped with a refrigerated autosampler (Tosohaas, Philadelphia, PA). The amines were separated on C-18 reversed phase column (3 mm X 100 mm) with flow rate of 1 ml/min isocratically. An LC 4B amperometric detector with glassy carbon electrode set at 0.65V versus an

Ag/AgCl reference electrode (Bioanalytical Systems Inc., West Lafayette, IN) was used to detect the catecholamines. Chromotograms were integrated using 427A integrator (Beckman, San Ramon, CA). The mobile phase consisted of monochloroacetic acid, 0.15M; sodium octyl sulfonate, 0.13mM; disodium EDTA, 0.67mM; sodium hydroxide 0.12M and 1.5% acetonitrile; pH was adjusted to 3.1 with phosphoric acid. The system was calibrated with norepinephrine (NE), epinephrine (EPI), 3,4- dihydroxyphenyl alanine (DOPA), 3,4- dihydroxyphenyl acetic acid (Dopac) and dopamine (DA). 3,4- Dihydroxy benzylamine (DHBA) was used as an internal standard. The main catecholamine released from PC12 cells by cyanide in our experiments was dopamine. Only small amounts of norepinephrine were released and no epinephrine was detected.

LD50 DETERMINATIONS: To evaluate the *in vitro* screen, test compounds were used in mice to protect against lethal doses of cyanide. Swiss Webster mice 21 to 26 g (Harlan Farms, Indianapolis, IN) were given standard laboratory chow and water *ad libitum*. They were maintained in a temperature/humidity controlled environment with a light-dark cycle 8 a.m. to 8 p.m. Potential antidotes were administered at specified times (Table 2) before sc doses of cyanide were given. Mortality was noted 24 hrs after cyanide treatment. LD_{50}s were calculated according to a commercially available computer program (Dose-effect analysis, J. Chou and T.C. Chau, Elsevier Science Publishers, Cambridge, England, 1985). Both LD_{50} and "r" values were obtained. The "r" values reflect the goodness of fit of the line connecting points of the dose-effect plots, 1.0 being a perfect fit.

RESULTS

Table 1 is a summary of all the results of the in vitro screening procedure. Significant observations made in each of the individual assays are discussed below.

ANTIOXIDANTS DEFENSE ENZYMES

Catalase and superoxide dismutase (SOD, CuZn form) are very susceptible to the action of cyanide. However, none of the 24 compounds screened in vitro prevented catalase inhibition by cyanide and only pyruvate blocked the inhibitory effect of cyanide on SOD. It should also be noted that hydrocortisone, naloxone, lazaroid and chlorpromazine partially protected SOD against cyanide inhibition.

The enzyme glutathione reductase is resistant to cyanide. In fact, to obtain any inhibition, 10 mM KCN had to be used. Nevertheless, valproate or pyruvate partially reversed the effect of cyanide on glutathione reductase.

INTRACELLULAR CALCIUM: About 2/3 of the potential antidotes tested partially prevented the rise in intracellular calcium caused by cyanide. This appears to be the most non-specific of the antidotal effects of the compounds tested. Imipramine, mannitol and naloxone were most effective, preventing between 60 and 70% of the cyanide-induced increase in intracellular free calcium.

PEROXIDE FORMATION: Prevention of peroxide generation by cyanide occurred with 1/3 of the compounds tested. Pyruvate was 100% effective whereas imipramine, bretylium and carbamazepine blocked between 61 and 75% of the effect. Alpha-ketoglutarate also decreased the amount of peroxides formed by over 50%.

Table 1. RANKINGS FOR COMPOUNDS SCREENED IN ARMY CONTRACT DAMD 17-89-C-9033

Compound	Inhib. Dopamine Release	Cytochrome Oxidase Inhibition	SOD Inhib.	GSH Reductase Inhib.	Catalase Inhib.	Peroxide Generation	Cytosolic Calcium	Total Score
Flunarizine	6.4	0	0	0	0	0	3	9.6
Phenytoin	10	0	0	0	0	0	1.8	11.8
-keto-glutarate	2.3	0	0	0	0	5.6	1.7	9.7
-tocopherol	0	0	0	0	0	0	1.4	1.4
Valproate	0	0	0	3.3	0	3.5	1.8	9.6
Allopurinol	10	0	0	0.2	0	0	2.4	12.6
Imipramine	0	0	0	1.6	0	7.5	6.4	15.5
Hydrocortisone	3.1	0	1.1	0.5	0	0	3.7	8.4
Mannitol	5.1	0	0	0.4	0	4.8	6.1	16.3
Naloxone	0	1.6	1.1	0.3	0	0	7.0	10
Pyruvate	0	7.0	3.3	3.3	0	10	-	23.6
Retinol Acetate	2.2	0	0	0.5	0	3.6	-	6.3
Lazaroid	0	0	1.1	0.2	0	0	-	1.3
Nifedipine	3.2	0	0	1.1	0	0	0	4.3
Bretylium	0	0	0.6	0	0	6.3	0	6.9
Carbamazepine	9.2	0	0.8	0	0	6.1	3.9	20
Chlorpromazine	0	0	1.1	0	0.07	0	0	1.2
Clonidine	0	0	0	0	0	0	0	0
Fructose	1.2	0	0	0.3	0	0	0	1.5
Glutathione	0	0	0.7	0.2	0.1	0	1.5	2.5
$MgCl_2$	0	0	0	0.4	0.23	0	0	0.6
Pentobarbital	0	0	0	0	0.1	0	0	0.1
Tolazoline	5	0	0	0.5	0.13	0	1.6	7.2
Ethanol	0	0	0	0	0	0	0	0

Ranking Scale = Values represent degree of blockade of the effets of cyanide. Complete reversal is assigned a value of 10, 80% reversal a value of 8, etc. The 3 antioxidant defense enzymes were grouped together and given a total value of 10 so that complete protection against the inhibitory effect of cyanide in any one enzyme system was assigned a value of 3.3.

Note that pyruvate, retinol acetate and lazaroid interferred with the calcium assay and no values for effects of these substances on cytosolic calcium are included in the table.

CYTOCHROME OXIDASE: Cyanide interacts strongly with cytochrome oxidase, so it is not surprising that only one substance, pyruvate, had any appreciable (70%) protective effect against cyanide inhibition of this enzyme. Partial protection (16%) was provided by naloxone, however.

DOPAMINE RELEASE: About 40% of the compounds tested inhibited dopamine release from PC12 cells by cyanide. Three substances were almost completely effective; phenytoin, allopurinol and carbamazepine. The calcium channel blockers, flunarizine and nifedipine inhibited the response, although the effect of nifedipine was weak.

Compounds which block the rise in cytosolic calcium should also block dopamine release, but no significant correlation was found between these parameters using the data of Table 1. However, other pharmacological effects of the substances listed in Table 1 may mask the correlation. Thus when imipramine and naloxone were omitted, a highly significant correlation ($p < 0.01$) was revealed (Figure 1). Imipramine blocks catecholamine reuptake (27) which might be expected to offset any inhibition of dopamine release. Naloxone, the opiate antagonist, also would tend to enhance catecholamine release since opiate receptors mediate inhibition of adrenal secretion (28,29). Thus it is reasonable to omit imipramine and naloxone from the linear regression shown in Figure 1. The highly significant correlation between blockade of cyanide-induced increases in cytosolic calcium and inhibition of dopamine release by cyanide supports the overall validity of the data.

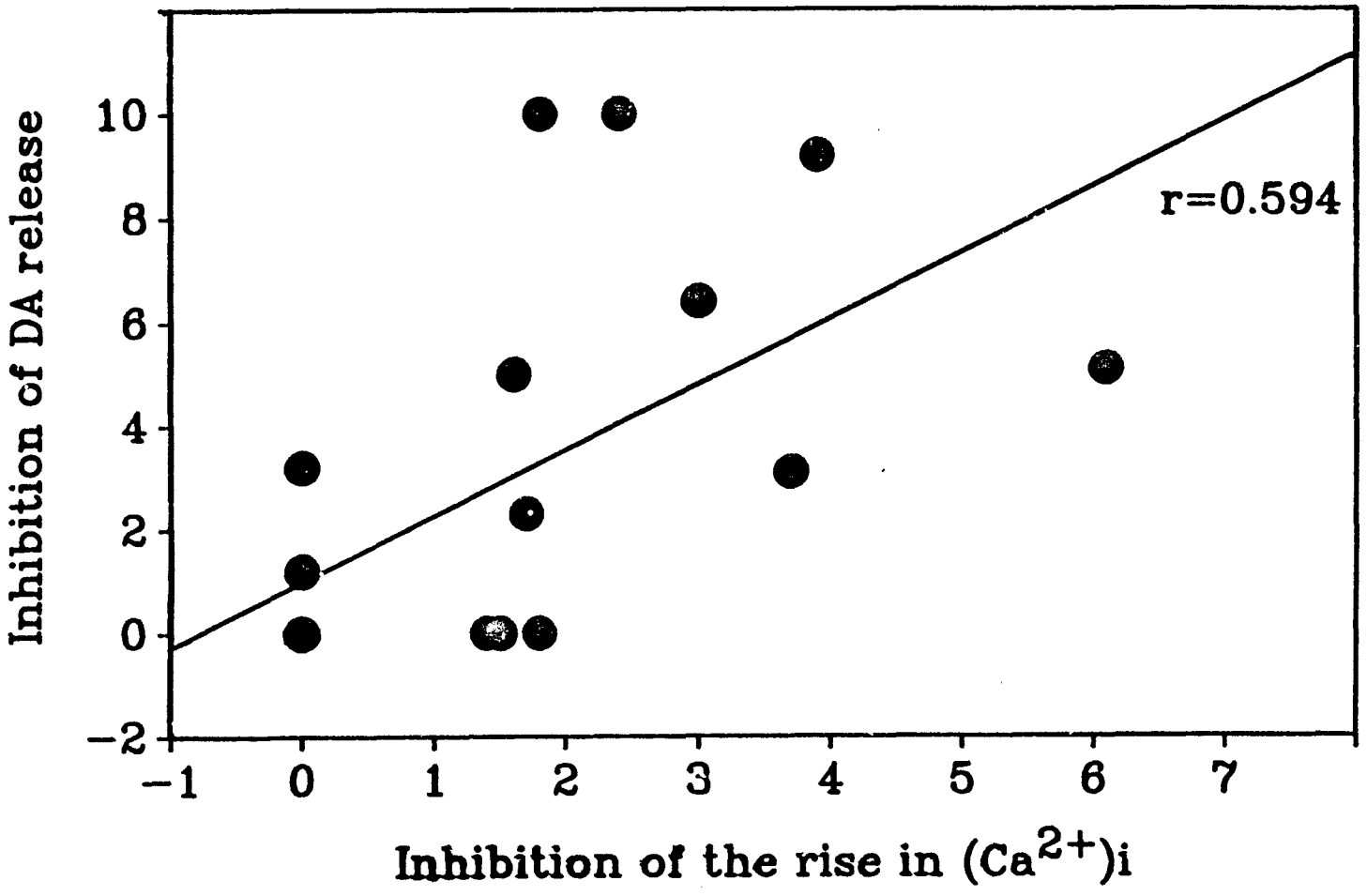
Inhibition of DA release
10
8
6
4
2
0
−2
−1
0
1
2
3
4
5
6
7
r=0.594
Inhibition of the rise in (Ca2+)i

Figure 1

LD50 DETERMINATION: Table 2 shows protection against lethal doses of cyanide by some of the test compounds. Also included for comparison are a few substances known to be effective antidotes for cyanide.

A plot of the protection against cyanide *in vivo* (LD_{50} values) and the test scores in the *in vitro* screen are shown in Figure 2. The value for alpha-ketoglutarate was omitted since its major antidotal action is that of a scavenger and only small amounts were used *in vitro* (10 μM) compared to *in vivo* (2 g/kg). The correlation coefficient of the remaining 6 points was 0.54 ($p < 0.27$). It appears from the available data involving a limited number of values that *in vitro* effectiveness may provide a good prediction of antidotal potency *in vivo*. Further testing is needed to establish the validity of the correlation.

DISCUSSION

Besides the correlation between *in vitro* and *in vivo* effectiveness against the effects of cyanide, the data provide some insight into biochemical mechanisms for cyanide intoxication as well as for possible antidotal actions.

One of the best antidotes for cyanide *in vivo* is alpha-ketoglutarate (30). This compound acts by chemically binding with cyanide at the ketone moiety producing an inactive cyanohydrin. The *in vitro* results revealed that alpha-ketoglutarate is also a good antioxidant. This antioxidant action may be important for the overall antidotal effectiveness of alpha-ketoglutarate.

Similarly, pyruvate is a cyanide scavenger but provides minimal protection against toxic doses of cyanide in mice (31).

Table 2

ANTIDOTE (route, dose, and time of administration before KCN)	LD50	"r" value
KCN alone	9.0	0.995
Scdium thiosulfate (ip, 1 g/kg, 15 min)	14.0	0.224
α-ketoglutarate (ip, 2 g/kg, 10 min)	35.3	0.977
Valproate (ip, 250 mg/kg, 15 min)	12.9	0.893
Sodium mercaptopyruvate (ip, 750 mg/kg, 15 min)	17.2	0.937
Phenoxybenzamine HCl (ip, 750 mg/kg, 15 min)	13.1	0.992
Sodium nitrite (sc, 100 mg/kg, 45 min)	27.8	0.910
Flunarizine HCl (ip, 30 mg/kg, 60 min)	9.1	0.609
Centrophenoxine (ip, 300 mg/kg, 20 min)	11.3	0.995
Allopurinol (ip, 100 mg/kg, 30 min)	10.7	0.917
Chlorpromazine HCl (ip, 10 mg/kg, 30 min)	10.0	0.917
Phenytoin (ip, 100 mg/kg, 30 min)	11.7	0.773

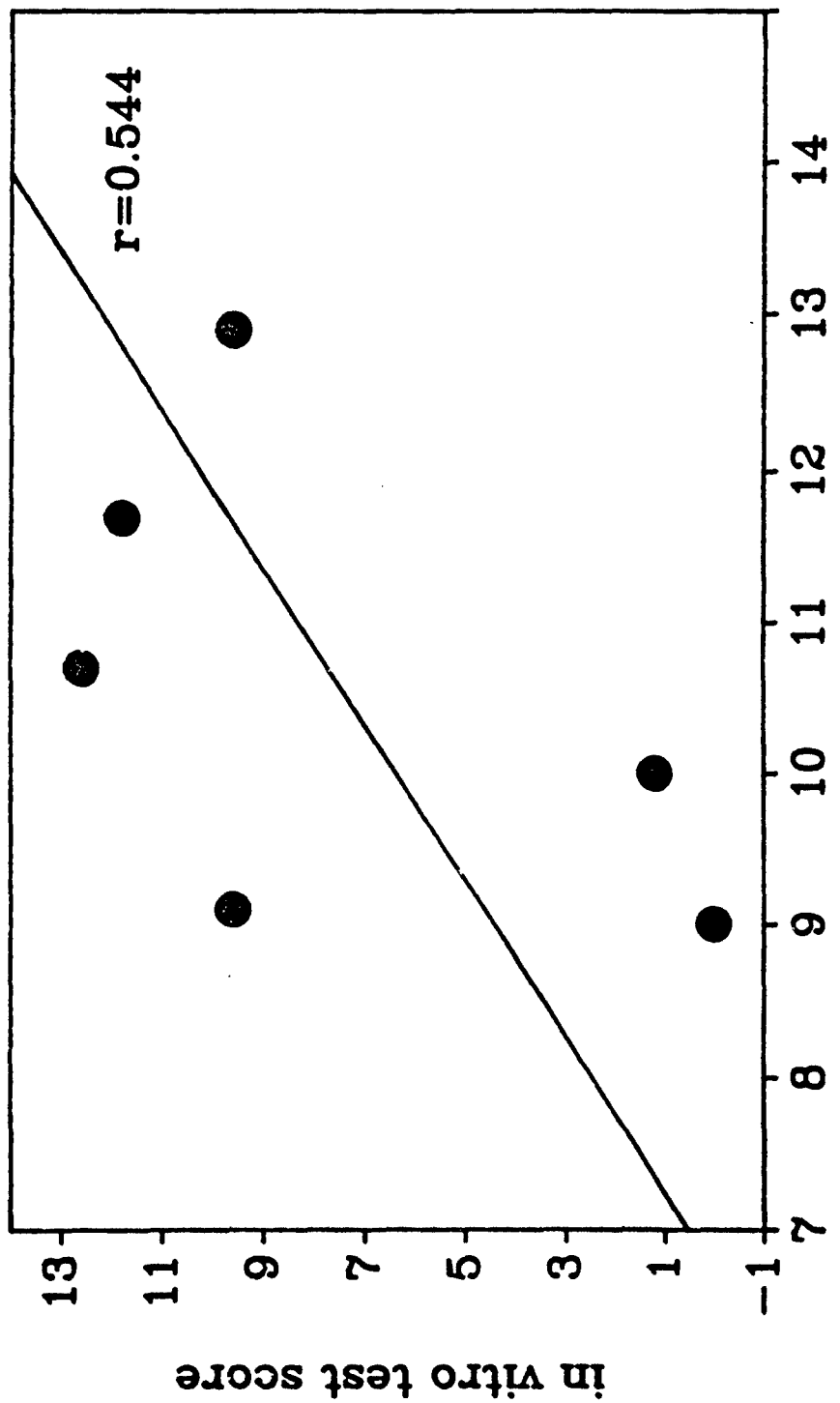

Figure 2

However pyruvate greatly enhances the effectiveness of sodium thiosulfate (increase in sodium cyanide LD_{50} from 19 to 38 mg/kg) (31). The *in vitro* screen shows that pyruvate is quite effective against oxidative mechanisms and completely protects superoxide dismutase and glutathione reductase against inhibition by cyanide. Furthermore, pyruvate quenched peroxide generation by cyanide better than any other substance tested. Pyruvate was also effective in preventing cytochrome oxidase inhibition by cyanide. The potent antioxidant action of pyruvate and its protection of cytochrome oxidase may explain pyruvate's ability to enhance thiosulfate antidotal effectiveness.

Cyanide intoxication in mice is antagonized by naloxone (32). Naloxone also blocks the rise in cytosolic calcium induced by cyanide in the *in vitro* screen of the present study. It is possible that the antidotal effect of naloxone may not be limited to antagonism of the opiate receptor.

The anticonvulsants, phenytoin and carbamazepine were both very effective in blocking cyanide-induced catecholamine release. Of all the 24 compounds tested, only allopurinol [which was recently reported to have anticonvulsant effects itself (33)] equalled the potency of of the anticonvulsants in this regard. The main anticonvulsant action of phenytoin and carbamazepine is thought to be blockade of sodium channels to inhibit high frequency discharges around epileptic foci (34). Minimal disruption of normal neuronal traffic is produced by these drugs. These results suggest that cyanide enhances sodium influx through channels responsible for high frequency discharges in convulsions. In

support, epileptiform discharges have been observed in guinea pig hippocampal slices after cyanide exposure in vitro (35).

An advantage of the in vitro screen is that it easily allows for testing the effects of drug combinations. The two most active compounds in the screening procedure employed in this study were pyruvate and carbamazepine. These substances may compliment one another very well biochemically. Pyruvate does not block dopamine release whereas carbamazepine is almost 100% effective in this regard. Pyruvate, but not carbamazepine, provides protection against the inhibitory effect of cyanide on cytochrome oxidase and two of the antioxidant defense enzymes. Both substances limit peroxide generation and carbamazepine inhibits the cyanide-induced rise in cytosolic calcium by about 40%. The combination of pyruvate and carbamazepine may prove to be quite effective in vitro as well as in vivo.

CONCLUSIONS

At the midpoint in Army Contract #DAMD17-89-C-9033, assays for seven biochemical effects of cyanide in cultured rat pheochromocytoma cells have been worked out to establish a screen for evaluation of potential antidotes. Twenty-four compounds have been tested in each of the seven assays and cyanide LD_{50} determinations in mice have allowed a preliminary correlation to be drawn between in vitro and in vivo effectiveness. One of the most active compounds tested in the in vitro screen was carbamazepine. It is anticipated that carbamazepine will also provide protection against cyanide toxicity in vivo. As more data become available, correlations will be drawn between protection in vivo and

inhibition of each of the individual biochemical changes produced by cyanide. It is possible that some of the biochemical changes detectable after cyanide exposure are unrelated to the lethal effects.

The two best cyanide antidotes found in the in vitro screening procedure were pyruvate and carbamazepine. These substances antagonize different biochemical effects of cyanide. Pyruvate is mainly an antioxidant but it also has the ability to partially reverse cyanide-induced inhibition of cytochrome oxidase. Carbamazepine on the other hand, inhibits the cyanide-induced rise in calcium as well as the evoked secretion of dopamine and furthermore limits cyanide-induced generation of peroxides. The combination of pyruvate and carbamazepine may be synergistic in protecting against cyanide-induced cellular damage. These substances should be tested together as cyanide antagonists in the in vitro screen as well as in vivo.

Evaluation of drug combinations as cyanide antidotes is complicated in regard to doses and time of administration in the mouse LD_{50} procedure and involves use of many animals. Blockade of cyanide-induced biochemical changes in isolated cells may be an efficient way to evaluate efficacy of combinations of substances against cyanide toxicity.

Cyanide intoxication is a complex syndrome and many factors probably contribute to the total cellular insult. It seems reasonable that substances which counteract the biochemical alterations induced by cyanide may be useful in supplementing the standard treatments involving a scavenger and a sulfur donor.

Thus, the best treatment for cyanide intoxication may involve a scavenger, a sulfur donor and biochemical antagonists.

In the second half of the contract period, the proposed work will be completed so that the in vitro screening procedure can be fully evaluated. In the process, emphasis will be placed on testing of drug combinations as cyanide antidotes.

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LEGENDS

Figure 1. Relationship Between Blockade of Cyanide-induced Increases in Cytosolic Calcium and Blockade of Cyanide-induced Dopamine Release from PC12 Cells. Nineteen different compounds from a variety of pharmacological or chemical classes were added to the incubation medium (0.01 mM) 15 min prior to stimulation with KCN (1 mM). Catecholamine release into the medium was measured using HPLC with electrochemical detection, and cytosolic calcium was measured using the fluorescent probe Quin 2. The correlation coefficient is 0.59 and the significance level of the correlation is $p < 0.01$.

Figure 2. Correlation Between *in vitro* and *in vivo* Effectiveness of Experimental Cyanide Antagonists. Drugs included in the above figure are cyanide alone, chlorpromazine, flunarizine, allopurinol, valproate and phenytoin. The relationship is not significant at the 5% level. The correlation coefficient is 0.54.